Mechanisms Underlying the Scratching Behavior Induced by the Activation of Proteinase-Activated Receptor-4 in Mice

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A role for proteinase-activated receptor-4 (PAR-4) was recently suggested in itch sensation. Here, we investigated the mechanisms underlying the pruriceptive actions of the selective PAR-4 agonist AYPGKF-NH_2 (AYP) in mice. Dorsal intradermal (i.d.) administration of AYP elicited intense scratching behavior in mice, which was prevented by the selective PAR-4 antagonist (pepducin P4pal-10). PAR-4 was found to be coexpressed in 32% of tryptase-positive skin mast cells, and AYP caused a 2-fold increase in mast cell degranulation. However, neither the treatment with cromolyn nor the deficiency of mast cells (WBB6F1-$Kit^{W/Wv}$ mice) was able to affect AYP-induced itch. PAR-4 was also found on gastrin-releasing peptide (GRP)-positive neurons (pruriceptive fibers), and AYP-induced itch was reduced by the selective GRP receptor antagonist RC-3095. In addition, AYP evoked calcium influx in ~1.5% of cultured DRG neurons also sensitive to TRPV1 (capsaicin) and/or TRPA1 (AITC) agonists. Importantly, AYP-induced itch was reduced by treatment with either the selective TRPV1 (SB366791), TRPA1 (HC-030031), or NK1 (FK888) receptor antagonists. However, genetic loss of TRPV1, but not of TRPA1, diminished AYP-induced calcium influx in DRG neurons and the scratching behavior in mice. These findings provide evidence that PAR-4 activation by AYP causes pruriceptive itch in mice *via* a TRPV1/TRPA1-dependent mechanism.

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INTRODUCTION

Itch is a common symptom of dermatological and systemic diseases such as atopic dermatitis and cholestasis (Ikoma *et al.*, 2006). Chronic itching remains without satisfactory treatment and lowers patients' quality of life (Weisshaar *et al.*, 2006). Studies using pruritogenic spicules obtained from cowhage (*Mucuna pruriens*) have revealed the existence of pruriceptive afferents distinct from the well-known histaminergic pathway (Davidson *et al.*, 2007; Johanek *et al.*, 2007; Namer *et al.*, 2008). The active component of cowhage is mucunain, a cysteine protease that acts as an activator of protease-activated receptors (PARs) (Shelley and Arthur, 1955; Reddy *et al.*, 2008). PARs are a subfamily of G-protein-coupled receptors, named PAR-1 to 4, that are activated by the proteolytic cleavage of their extracellular domain (Vergnolle, 2009).

With the discovery of PAR-2 involvement in itch, great progress has been made in terms of understanding the pathophysiological basis of itching (Steinhoff *et al.*, 2003). PAR-2 (Costa *et al.*, 2008, 2010) and, more recently, PAR-4 (Kempkes *et al.*, 2014) were suggested to mediate itch. PAR-4 is expressed on rodent sensory neurons (Asfaha *et al.*, 2007; Auge *et al.*, 2009) and can be activated by several endogenous proteinases and synthetic hexapeptides (Fu *et al.*, 2014). Interestingly, the itch-causing agent mucunain cleaves PAR-4 more potently than PAR-2 (Reddy *et al.*, 2008). Furthermore, it was shown that cathepsin S, an endogenous cysteine protease that shares sequence homology with the mucunain active site, evokes itch in humans via activation of both PAR-2 and PAR-4 (Reddy *et al.*, 2010). Indeed,

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Abbreviations: AYP, AYPGKF-NH_2; DRG, dorsal root ganglion; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor; PAR-4, proteinase-activated receptor-4; SP, Substance P; TRP, transient receptor potential; TRPA1, transient receptor potential ankyrin-1; TRPV1, transient receptor potential vanilloid-1; BBB, blood–brain barrier

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intradermal (i.d.) injection of PAR-4 agonists caused scratching behavior in mice (Tsujii *et al.*, 2008; Akiyama *et al.*, 2009, 2010).

Although evidences suggest a role for PAR-4 in itch, the signaling mechanisms involved in this process are poorly understood. Here we investigated the cellular and molecular mechanisms associated with the scratching behavior induced by the PAR-4-activating peptide AYPGKF-NH_2 (AYP) in mice, and provided data supporting the role of PAR-4 in itch. We show that AYP elicits scratching behavior in mice by activating transient receptor potential (TRP) channels and possibly causing the release of itch-mediating neurotransmitters. These findings highlight the potential of PAR-4 as a target for the development of antipruritic drugs.

RESULTS

PAR-4 activation induces scratching behavior in mice

I.d. administration of the selective PAR-4 agonist AYP, but not the inactive peptide YAPGKF-NH_2 (YAP), elicited scratching behavior when injected into the back of the mouse neck (Figure 1a) with an effective dose ranging from 100 to 500 nmol per site and an estimated mean ED_{50} value (accompanied by 95% confidence limit) of 156 (42–572) nmol per site. The dose of 200 nmol per site was chosen for all the subsequent experiments. AYP-induced scratching behavior was time-dependent, peaking within 10 minutes and decreasing slowly over time, without a significant response at 30 minutes (Figure 1b). Interestingly, the number and the time-course profiles of AYP-evoked scratching bouts were similar to those caused by histamine (Figures 1a and b), a widely known pruritogenic agent. As expected, pretreatment with the selective PAR-4 antagonist pepducin P4pal-10 significantly reduced AYP-induced scratching behavior (Figure 1c). In addition, pretreatment with the nonselective opioid receptor antagonist naloxone, used as an antipruritic control drug, also significantly inhibited AYP-induced response (Figure 1d).

AYP-induced scratching behavior is dependent on GRP-expressing fibers

We found PAR-4 to be expressed in ~32% of all mouse skin mast cells and AYP (200 nmol/site) i.d. injection was able to cause mast cell degranulation; however, AYP-induced itch was not dependent on mast cell product release (Supplementary results and Supplementary Figure S1 online). In addition, we detected PAR-4 on skin sensory neurons as PAR-4 immunoreactivity was colocalized with the neuronal marker PGP 9.5 (Figures 2a and b). PAR-4 was also found in the soma of 47% of mouse dorsal root ganglion (DRG) neurons (247/525). Of those, 39% (95/247), 34% (85/247), and 27% (67/247) were small-, medium-, and large-diameter neurons, respectively. To investigate the phenotype of PAR-4-

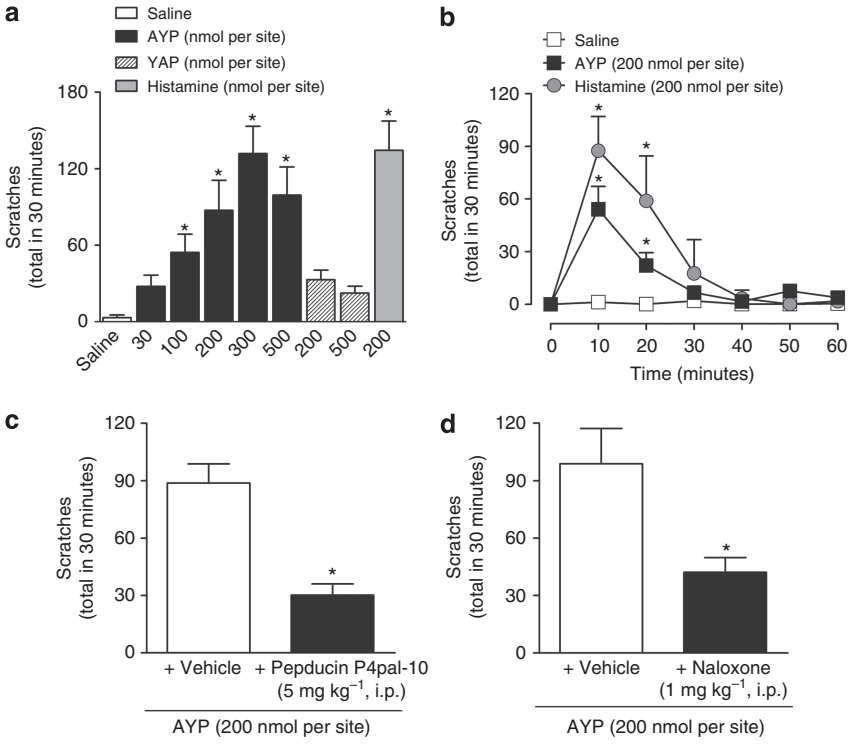

Figure 1. Intradermal injection of AYPGKF-NH_2 (AYP) causes itch-like behavior. (**a**) Scratching behavior elicited by AYP (30–500 nmol per site), histamine (200 nmol per site), or control peptide YAP (200–500 nmol per site). (**b**) Time-course profile of scratching behavior induced by AYP (200 nmol per site) or histamine (200 nmol per site). Effect of (**c**) the proteinase-activated receptor-4 (PAR-4) antagonist pepducin P4pal-10 (5 mg kg^{-1}, i.p., 60 min) and (**d**) the opioid receptor antagonist naloxone (1 mg kg^{-1}, i.p., 30 min) on AYP (200 nmol per site)-induced scratching. Each column represents the mean of 6–8 animals, and the vertical bars represent the SEM. Significant differences (*$P<0.05$) were indicated, as compared with the (**a**, **b**) saline- or (**c**, **d**) vehicle-treated group. (**a**) One-way analysis of variance (ANOVA) followed by Bonferroni's test. (**b**) Two-way ANOVA followed by Bonferroni's test. (**c**, **d**) Student's *t*-test.

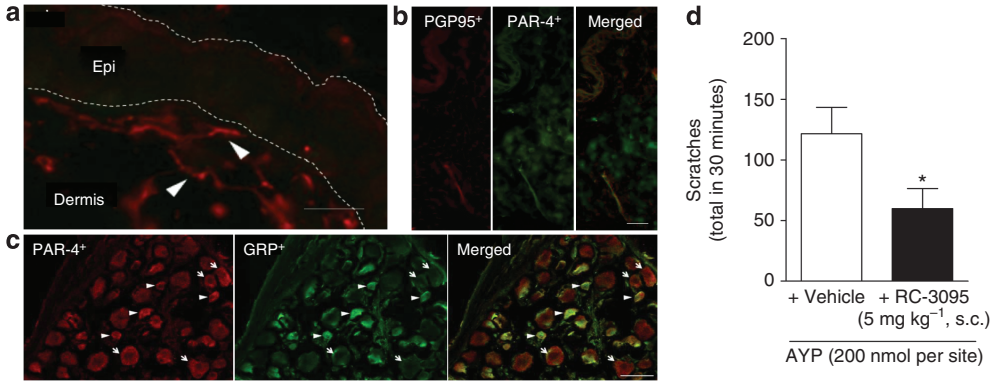

Figure 2. Scratching behavior elicited by AYPGKF-NH_2 (AYP) is dependent on GRP-expressing neurons. (**a**) proteinase-activated receptor-4 (PAR-4) immunoreactivity in dermal nerve fibers. Scale bar, 10 μm. Colocalization of PAR-4 with (**b**) the neuronal marker protein gene product 9.5 (PGP 9.5) in mouse skin sections or (**c**) the pruriceptive neuron marker gastrin-releasing peptide (GRP) in mouse DRG sections. Arrowheads: $PAR\text{-}4^+/GRP^+$. Arrows: $PAR\text{-}4^+/GRP^-$. Scale bar, 50 μm. (**d**) Effect of the GRP receptor antagonist RC-3095 (5 mg kg^{-1}, s.c., 60 min) on AYP (200 nmol per site)-induced scratching behavior. Each column represents the mean of 6 animals, and the vertical bars represent SEM. Significant differences ($*P<0.05$) were indicated, as compared with the vehicle-treated group. Student's *t*-test. epi: epidermis.

expressing neurons, we performed a double labeling for PAR-4 and gastrin-releasing peptide (GRP), a marker of pruriceptive neurons (Sun and Chen, 2007). Interestingly, 77% (191/247) of all PAR-4-positive cells were colocalized with GRP, and 87% of the GRP-positive cells (184/212) expressed PAR-4 (Figure 2c). The functional relevance of GRP-containing neurons for PAR-4-mediated scratching behavior was then investigated in animals pretreated with the selective GRP receptor (GRPR) antagonist RC-3095. Of relevance, scratching behavior was significantly prevented by RC-3095 administration (Figure 2d).

AYP stimulates TRP channel–expressing DRG neurons

To determine whether AYP has direct effects on primary sensory neurons, we performed live-cell calcium imaging. Interestingly, AYP (200 μM) evoked an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in only 1.5% (170/11,273) of the DRG neurons from wild-type mice (Figure 3). To investigate whether PAR-4 was localized to TRP channel–expressing DRG neurons, cells were challenged sequentially with AYP (200 μM), the TRPA1 agonist allyl isothiocyanate (AITC; 50 μM), and the TRPV1 agonist capsaicin (1 μM), followed by KCl (50 mM). Of all AYP-sensitive neurons, 54.2% (70/129) were responsive to both capsaicin and AITC (Figure 3a), whereas 31% (40/129) and 11.6% (15/129) were responsive to only capsaicin (Figure 3b) or AITC (Figure 3c), respectively. Consequently, 96.9% (125/129) of the AYP-sensitive neurons were sensitive to either capsaicin or AITC, and 3.1% (4/129) of AYP-responsive neurons were insensitive to these agonists.

We next examined the responses of DRG neurons from TRPV1 ($Trpv1^{-/-}$) or TRPA1 ($Trpa1^{-/-}$) knockout mice to AYP. Notably, the responses to AYP were eliminated in the $Trpv1^{-/-}$ neurons in comparison with neurons from wild-type animals (0/2,363 neurons; $P<0.0001$, Chi-squared test). However, no reduction was observed in the percentage of AYP-responsive neurons from $Trpa1^{-/-}$ mice (1.97%; 54/2,745 neurons) where the proportion of AYP-responsive neurons was slightly higher than in wild-type neurons ($P<0.05$, Chi-squared test). Notably, almost all AYP-sensitive $Trpa1^{-/-}$ neurons (53/54) responded to capsaicin, consistent with the findings in preparations from wild-type mice (Figure 3d).

PAR-4-mediated scratching behavior requires TRP channel activation and SP release

We investigated the functional involvement of TRP channels in AYP-induced scratching behavior. TRPV1 and TRPA1 are expressed in sensory afferents neurons and are involved in itch transmission (Imamachi *et al.*, 2009; Wilson *et al.*, 2011). The importance of these channels was assessed in animals pretreated with either the selective TRPV1 (SB366791) or TRPA1 (HC-030031) receptor antagonists. Results show that both antagonists were able to alleviate AYP-induced scratching behavior by 61% and 55%, respectively (Figures 4a and b). Similarly, itch was attenuated in AYP-treated $Trpv1^{-/-}$ mice in comparison with their WT counterparts (Figure 4c). In contrast, TRPA1 deletion appeared to increase the scratching response evoked by AYP, although this was not statistically significant (Figure 4c).

Substance P (SP), a neuropeptide found in TRPV1- and TRPA1-positive fibers, mediates itch by activating NK_1 receptors (Steinhoff *et al.*, 2006; Tey and Yosipovitch, 2011). Accordingly, pretreatment with the selective NK_1 receptor antagonist FK888 significantly reduced the number of AYP-induced scratching bouts (52% inhibition; Figure 4d).

DISCUSSION

In the present study, we provide evidence that PAR-4 mediates scratching behavior in mice by activating TRP channels and possibly causing the release of GRP and SP. A role for PAR-4 in itch was first suggested by Tsujii and collaborators (2008) who demonstrated that AYP elicits scratching behavior in mice at the dose of 100 nmol/site.

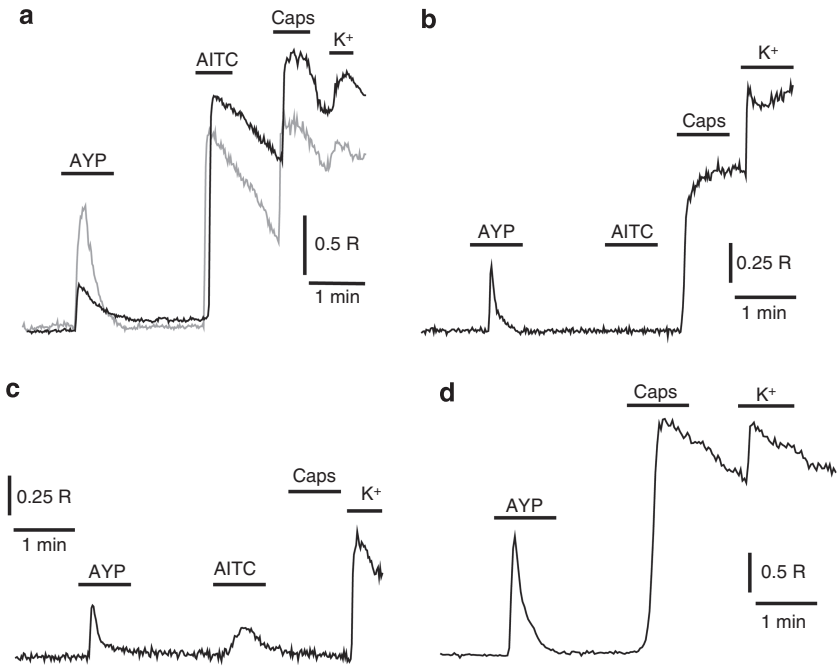

Figure 3. AYPGKF-NH_2 (AYP) evoked increases in $[Ca^{2+}]_i$ in dorsal root ganglion (DRG) neurons from wild-type but not *Trpv1*$^{-/-}$ mice. (**a**) Typical recordings of AYP (200 μM) evoked increases in $[Ca^{2+}]_i$ in wild-type DRG neurons that were sensitive to AITC and capsaicin, indicative of transient receptor potential ankyrin-1 (TRPA1) and transient receptor potential vanilloid-1 (TRPV1) coexpression. (**b**) AYP (200 μM)-responsive DRG neuron that was also responsive to only capsaicin. (**c**) AYP (200 μM)-responsive DRG neuron that was also responsive to only AITC. (**d**) Example of DRG neurons from *Trpa1*$^{-/-}$ mice that responded to AYP and capsaicin.

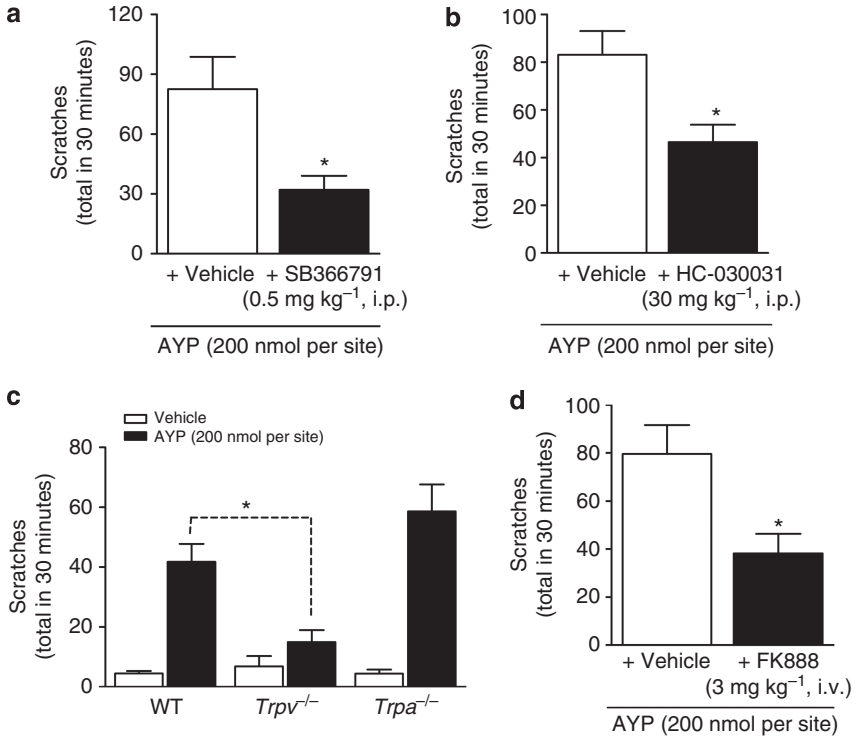

Figure 4. AYPGKF-NH_2 (AYP)-induced itch-like behavior was dependent on transient receptor potential (TRP) activation and Substance P (SP) release. Effects of (**a**) the transient receptor potential vanilloid-1 (TRPV1) antagonist SB366791 (0.5 mg kg^{-1}, i.p., 30 min), (**b**) the transient receptor potential ankyrin-1 (TRPA1) antagonist HC-030031 (30 mg kg^{-1}, i.p., 30 min), or (**d**) the NK1 receptor antagonist FK888 (3 mg kg^{-1}, i.v., 15 min) on AYP (200 nmol per site)-elicited scratching behavior. (**c**) Scratching behavior induced by AYP (200 nmol per site) in TRPV1 (*Trpv1*$^{-/-}$) and TRPA1 (*Trpa1*$^{-/-}$) knockout mice. Each column represents the mean of six animals, and the vertical bars represent SEM. Significant differences (*$P<0.05$) were indicated, as compared with (**a**, **b**, **d**) the vehicle-treated group or (**c**) the wild-type (WT) mice group. (**a**, **b**, **d**) Student's *t*-test. (**c**) One-way ANOVA followed by Bonferroni's test.

Curiously, in a study conducted by the same group in 2009, AYP at the same dose, failed to cause a pruriceptive response in mice (Tsujii *et al.*, 2009). These discrepant results may be owing to the use of different strains of mice of different ages in these studies. Here, scratching behavior was elicited by AYP injection in the nape of the neck, and it was inhibited by the selective PAR-4 antagonist (confirming AYP selectivity) and by naloxone, a centrally acting opioid receptor antagonist used to treat some clinical itch conditions (Phan *et al.*, 2010). Indeed, AYP-induced hindpaw scratching bouts had been previously described in the same model (Akiyama *et al.*, 2009) and later confirmed in the cheek model of itch (Akiyama *et al.*, 2010) and in the alloknesis model (Akiyama *et al.*, 2012).

Both human and rat mast cells can express PAR-4 (Han *et al.*, 2011; Russell *et al.*, 2011), and mast cells are considered to be central in some itching conditions (Steinhoff *et al.*, 2006). However, the contribution of histamine to AYP-induced itch is rather controversial, with evidence suggesting this response to be dependent on (Tsujii *et al.*, 2008) and independent of (Akiyama *et al.*, 2009; Tsuji et al., 2009) this pathway. Here, we show that PAR-4 is also expressed on mouse mast cells and that AYP has the ability to cause their degranulation. However, we present evidence that AYP elicits itch in a mast cell–independent manner, with neither mast cells nor their mediators playing a role in this response. It is important to highlight that the discrepancies observed between our study and previous studies addressing the contribution of histamine to AYP-induced itch may be related to differences in mouse strain, gender and/or age, and differences in AYP dose; in addition to the use of different strategies to investigate histamine contribution to this response.

We also present evidence that PAR-4 is expressed on sensory neurons innervating the mouse skin, as confirmed by its colocalization with the neuronal marker PGP 9.5. PAR-4 was previously detected on the rat DRG neurons colocalized with neuropeptides such as SP (Asfaha *et al.*, 2007). Herein, PAR-4 was found in the soma of small- to large-sized DRG neurons. We found that 77% of all PAR-4-positive neurons contain the pruriceptive marker GRP and 87% of all GRP-positive cells also express PAR-4. GRP can be found in peptidergic fibers innervating the mouse skin (Tominaga *et al.*, 2009) and in small- to medium-sized DRG neurons, which also express SP and TRPV1 (Sun and Chen, 2007). Although still debatable (Bautista *et al.*, 2014), GRP-dependent pruriceptive pathway is composed of peptidergic C fibers containing GRP and by GRPR in the spinal cord. The genetic deletion or antagonism of GRPR, as well as the depletion of GRP-positive fibers, was able to prevent pruritus in mice (Sun and Chen, 2007; Sun *et al.*, 2009). Corroborating these findings, the systemic treatment with the selective GRPR antagonist RC-3095 prevented AYP-induced scratching behavior, suggesting that this response involves the release of GRP. This compound is known for its ability to cross the blood–brain barrier (BBB) (Andoh *et al.*, 2011); thus, it is possible that spinal cord–located GRPR contribute to AYP-induced itch.

We hypothesized that AYP induces itch by activating PAR-4 on skin pruriceptive fibers with cellular bodies in the DRG. Surprisingly, calcium imaging experiments showed that AYP evokes calcium influx in only ~1.5% of the total cultured DRG neurons (small to medium diameter), contrasting with the broad expression pattern of PAR-4 in small- to large-diameter neurons (47%). This discrepancy may be owing to the different approaches used in this study—i.e., immunohistochemistry (*ex vivo) versus* calcium imaging (*in-vitro*)—as protein expression does not always translate in to functional responses. Of all AYP-responsive neurons, ~31% and ~11% were, respectively, responsive to only the TRPV1 agonist or the TRPA1 agonist. In addition, ~54% of the AYP-sensitive neurons were sensitive to both compounds. Thus, of all PAR-4-expressing DRG neurons, ~85% express TRPV1, ~61% express TRPA1, and ~54% express both channels. Next, we stimulated cultured *Trpv1*$^{-/-}$ and *Trpa1*$^{-/-}$ mouse DRG neurons with AYP and found that AYP response is lost in *Trpv1*$^{-/-}$ but not in *Trpa1*$^{-/-}$-derived DRG neurons. The absence of any AYP-responsive DRG neurons from *Trpv1*$^{-/-}$ mice is surprising given that a small percentage of the AYP-responsive neurons appeared to be sensitive to AITC but not to capsaicin. However, in these experiments, wild-type neurons were challenged with AITC before capsaicin, raising the possibility that AITC either desensitized TRPV1 or masked any subsequent response that may have been evoked by capsaicin.

Current studies have reported both additional and contrary roles for TRPV1 and TRPA1 channels in the response to different pruritogens (Wilson *et al.*, 2011; Fernandes *et al.*, 2013). We investigated the *in vivo* contribution of TRPV1 and TRPA1 receptors to AYP-induced itch. Although TRPV1 deletion or antagonism (by SB366791) clearly diminished this response, conflicting results were found when assessing the role of TRPA1 in this model. Indeed, treatment with the selective TRPA1 antagonist HC-030031 reduced PAR-4-mediated itch. However, no reduction in scratching behavior was observed in *Trpa1*$^{-/-}$ mice and the response appeared to be augmented. It is possible that these different results are due to compensatory mechanisms in *Trpa1*$^{-/-}$ mice. Interestingly, although the sample sizes are small, the percentage of AYP-responsive DRG neurons was slightly higher in preparations from *Trpa1*$^{-/-}$ than from wild-type mice. Indeed, the existence of compensatory mechanisms in TRPA1$^{-/-}$ mice was previously suggested in a study by Petrus and collaborators (2007). This study compared the hyperalgesic responses of TRPA1$^{-/-}$ mice with those treated with a selective TRPA1 antagonist AP18. AP18-treated mice exhibited less CFA-induced hyperalgesia than mice lacking TRPA1. The authors attributed the compensatory changes in TRPA1$^{-/-}$ mice to possible changes in TRPV4 but not TRPV1 expression in skin cells and DRG, a hypothesis that remains unclear.

Although a central effect was suggested for SB366791 following its systemic administration (Fernandes *et al.*, 2011), to our knowledge, no studies have investigated the ability of HC-030031 to cross the blood–brain barrier when systemically administered. However, it is possible that based on the pharmacologic properties of HC-030031 (see the PubChem

Public Chemical Database) this compound may be able to penetrate the BBB, although the extent to which this happens is not completely known at this time. Thus, these drugs may be acting at both central and peripheral levels in order to inhibit AYP-induced itch. Indeed, in addition to afferent sensory fibers, TRPV1 and TRPA1 are also expressed on skin mast cells and keratinocytes (Biro and Kovacs, 2009; Buch *et al.*, 2013; Oh *et al.*, 2013). Although a neuronal role has been proposed for TRP channels in itch, non-neuronal TRPA1 was recently implicated in this phenomenon (Fernandes *et al.*, 2013). Thus, we can suggest that TRPV1/TRPA1-expressing pruriceptive fibers, as well as skin non-neuronal cells, mediate AYP-induced scratching behavior.

SP is a neuropeptide present in TRPV1/TRPA1-expressing sensory neurons, and it has been implicated in itch transmission through its NK1 receptor at both peripheral and spinal sites (Andoh *et al.*, 1998; Akiyama *et al.*, 2010). Here, we show that AYP-induced itch is reduced by NK1 receptor antagonism (by FK888), suggesting that SP is released upon sensitization/activation of TRP channels by the PAR-4 agonist. Indeed, SP was found to be expressed on pruriceptive neurons together with TRPV1 or TRPA1 channels (Sun and Chen, 2007; Imamachi *et al.*, 2009; Liu *et al.*, 2009; Wilson *et al.*, 2013). SP release may occur at both peripheral and central levels, as TRPV1 activation can cause its release at both sites and FK888 was suggested to cross the BBB (Rudd *et al.*, 1999; Willcockson *et al.*, 2010; Steagall *et al.*, 2012).

Taken together, our findings suggest that itch-like behavior induced by PAR-4 agonist is dependent on the action of AYP on skin sensory neurons and perhaps on non-neuronal cells. In turn, this may trigger the activation of TRPV1 and TRPA1 channels, as well as the possible release of SP and GRP, transmitting the pruriceptive signal to the CNS. Overall, our data suggest that PAR-4-selective antagonists can constitute interesting tools for the attenuation of chronic itch, which remains a challenge to treat in the clinical practice. However, future studies addressing the intracellular pathways connecting the activation of PAR-4 and TRPV1/TRPA1 sensitization are of importance to further understand the pathophysiology of itch, and remain to be investigated.

MATERIALS AND METHODS

Animals

Female adult CD1 mice (8–10 weeks old) from the Universidade Federal de Santa Catarina were used. In addition, female mice (8–12 weeks old) either WT C57BL/6J, TRPV1-knockout (*Trpv1*$^{-/-}$; C57BL/6 background (Costa *et al.*, 2008)), or TRPA1-knockout (*Trpa1*$^{-/-}$; C57BL/6 J background (Andersson *et al.*, 2013)) were used. Mice were kept in a climatically controlled environment with *ad libitum* access to food and water and were acclimatized in the procedure room for 1 hour before the experiments. Experimental procedures were approved by the local ethics committee of the Universidade Federal de Santa Catarina and King´s College London, and were performed in accordance with the National Institutes of Health Animal Care Guidelines (NIH publications nº.80-23) and the UK Home Office Animals (Scientific Procedures) Act of 1986.

Scratching behavior

The experiments were performed as previously described (Costa *et al.*, 2010). Mice received a dorsal i.d. injection of either vehicle (saline; 50 µl per site), AYP (30–500 nmol per site), YAP (200 or 500 nmol per site), or histamine (200 nmol per site). Scratching behavior was observed for either 30 or 60 minutes, and quantified as the number of scratches made with the mouse hindpaws near the injected site. The results are expressed as the number of scratches in 30 minutes or in intervals of 10 minutes.

Immunohistochemistry analysis

PAR-4 expression in the mouse skin and on DRG neurons was assessed by immunofluorescence staining followed by confocal microscopy. Anesthetized animals were perfused with saline followed by 4% PFA phosphate buffer. Mouse rostral back skin samples (~1.5 cm^2) and DRGs from cervical and thoracic regions were collected. Skin samples were embedded in paraffin and sectioned (4 µm). DRG samples were fixed in 4% PFA, cryopreserved in 30% sucrose, embedded in Tissue-Tek (Sakura Finetek, Torrance, CA), and sectioned (16 µm) at −15 °C on a cryostat (Leica, Heidelberg, Germany). Slices were incubated overnight at 4 °C with PAR-4 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), protein gene product 9.5 (PGP 9.5) (1:100; Santa Cruz Biotechnology), or GRP (1:1000; Immunostar, Hudson, WI) antibodies, followed by incubation with secondary antibodies conjugated to Alexa Fluor-568 (1:750) or Alexa Fluor-488 (1:400) (Molecular Probes, Invitrogen, New York, NY) at room temperature for 3 h. A series of images from different focal planes within the DRG section were collected into a single file (e.g., z-series), using confocal microscopy (Leica). In DRG slices, the number of PAR-4$^+$, GRP$^+$, or PAR-4$^+$/GRP$^+$ neurons was determined with the NIH ImageJ 1.36b imaging software (NIH, Bethesda, MD). Images from three random fields per section of skin samples were evaluated and captured as described above.

Cell culture and intracellular [Ca^{2+}] measurements

DRG neurons were obtained and prepared from adult WT, *Trpv1*$^{-/-}$, or *Trpa1*$^{-/-}$ female C57BL6/J mice, as previously described (Bevan and Winter, 1995; Andersson *et al.*, 2012). Isolated neurons were cultured in MEM supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM L-glutamine, and 50 ng ml^{-1} NGF (Promega, Southampton, UK) for less than 24 hours before experimentation.

Cultured DRG neurons were loaded with 2.5 µM Fura-2 AM (Molecular Probes, Paisley, UK) in the presence of 1 mM probenecid for ~1 hour. The dye loading and subsequent experiments were performed in a saline solution (pH 7.4) containing (in mM) 140 NaCl, 5 KCl, 10 glucose, 10 HEPES, 2 $CaCl_2$, and 1 $MgCl_2$. AYP (200 µM) was applied to cells by local continuous microperfusion of this solution through a fine tube placed very close to the cells being studied. TRP channel expression in individual neurons was tested functionally by sequential application of the agonists for TRPV1 (capsaicin, 1 µM; Sigma-Aldrich, UK) and TRPA1 (allyl isothiocyanate (AITC), 50 µM; Sigma-Aldrich, UK). All neurons were finally identified by the increase in intracellular calcium concentration evoked by depolarization with 50 mM KCl. Experiments were conducted at 35 °C. Images of a group of cells were captured every 1–2 seconds using 340- and 380-nm excitation wavelengths with emission measured at >510 nm with a microscope-based imaging system

(PTI, New Jersey, NJ). Analyses of emission intensity ratios at 340 nm/380 nm excitation (R, in individual cells) were performed using the Image Master suite of software.

Drugs

AYPGKF-NH_2 (AYP), YAPGKF-NH_2 (YAP), and N-palmitoyl-SGRRY-GHALR-NH_2 (P4pal-10; GenScript, Piscataway, NJ,); naloxone (Research Biochemicals International, Natick, MA); histamine, RC--3095, and SB366791(Sigma-Aldrich, St Louis, MO); FK888 (donated by the Fujisawa Pharmaceutical, Osaka, Japan); and HC-030031 (synthesized at the Universidade Federal de Santa Catarina, as previously described (Moram *et al.*, 2007)) were used. All drugs were dissolved in saline, except HC-030031 and FK888. HC-030031 stock solution (10^{-1} M) was prepared in saline with 90% dimethylsulfoxide and 10% Tween 80. FK888 stock solution (10^{-3} M) was prepared in saline containing 5% ethanol. For *in vivo* experiments, the final concentrations of dimethylsulfoxide and ethanol did not exceed 5% and 1%, respectively.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Akiyama T, Carstens MI, Carstens E (2010) Differential itch- and pain-related behavioral responses and micro-opoid modulation in mice. *Acta Derm Venereol* 90:575–81

Akiyama T, Carstens MI, Ikoma A *et al.* (2012) Mouse model of touch-evoked itch (alloknesis). *J Invest Dermatol* 132:1886–91

Akiyama T, Merrill AW, Zanotto K *et al.* (2009) Scratching behavior and Fos expression in superficial dorsal horn elicited by protease-activated receptor agonists and other itch mediators in mice. *J Pharmacol Exp Ther* 329: 945–51

Andersson DA, Gentry C, Bevan S (2012) TRPA1 has a key role in the somatic pro-nociceptive actions of hydrogen sulfide. *PloS One* 7:e46917

Andersson DA, Gentry C, Light E *et al.* (2013) Methylglyoxal evokes pain by stimulating TRPA1. *PloS One* 8:e77986

Andoh T, Kuwazono T, Lee JB *et al.* (2011) Gastrin-releasing peptide induces itch-related responses through mast cell degranulation in mice. *Peptides* 32:2098–103

Andoh T, Nagasawa T, Satoh M *et al.* (1998) Substance P induction of itch-associated response mediated by cutaneous NK1 tachykinin receptors in mice. *J Pharmacol Exp Therap* 286:1140–5

Asfaha S, Cenac N, Houle S *et al.* (2007) Protease-activated receptor-4: a novel mechanism of inflammatory pain modulation. *Br J Pharmacol* 150: 176–85

Auge C, Balz-Hara D, Steinhoff M *et al.* (2009) Protease-activated receptor-4 (PAR 4): a role as inhibitor of visceral pain and hypersensitivity. *Neurogastroenterol Motil* 21:1189–e107

Bautista DM, Wilson SR, Hoon MA (2014) Why we scratch an itch: the molecules, cells and circuits of itch. *Nat Neurosci* 17:175–82

Bevan S, Winter J (1995) Nerve growth factor (NGF) differentially regulates the chemosensitivity of adult rat cultured sensory neurons. *J Neurosci* 15: 4918–26

Biro T, Kovacs L (2009) An "ice-cold" TR(i)P to skin biology: the role of TRPA1 in human epidermal keratinocytes. *J Investig Dermatol* 129:2096–9

Buch TR, Schafer EA, Demmel MT *et al.* (2013) Functional expression of the transient receptor potential channel TRPA1, a sensor for toxic lung inhalants, in pulmonary epithelial cells. *Chem Biol Interact* 206:462–71

Costa R, Manjavachi MN, Motta EM *et al.* (2010) The role of kinin B1 and B2 receptors in the scratching behaviour induced by proteinase-activated receptor-2 agonists in mice. *Br J Pharmacol* 159:888–97

Costa R, Marotta DM, Manjavachi MN *et al.* (2008) Evidence for the role of neurogenic inflammation components in trypsin-elicited scratching behaviour in mice. *Br J Pharmacol* 154:1094–103

Davidson S, Zhang X, Yoon CH *et al.* (2007) The itch-producing agents histamine and cowhage activate separate populations of primate spinothalamic tract neurons. *J Neurosci* 27:10007–14

Fernandes ES, Russell FA, Spina D *et al.* (2011) A distinct role for transient receptor potential ankyrin 1, in addition to transient receptor potential vanilloid 1, in tumor necrosis factor alpha-induced inflammatory hyperalgesia and Freund's complete adjuvant-induced monarthritis. *Arthritis Rheum* 63:819–29

Fernandes ES, Vong CT, Quek S *et al.* (2013) Superoxide generation and leukocyte accumulation: key elements in the mediation of leukotriene B (4)-induced itch by transient receptor potential ankyrin 1 and transient receptor potential vanilloid 1. *FASEB J* 27:1664–73

Fu Q, Cheng J, Gao Y *et al.* (2014) Protease-activated receptor 4: a critical participator in inflammatory response. *Inflammation* 38:886–95

Han W, Wang Z, Lu X *et al.* (2011) Protease activated receptor 4 status of mast cells in post infectious irritable bowel syndrome. *Neurogastroenterol Motil* 24:113–9

Ikoma A, Steinhoff M, Stander S *et al.* (2006) The neurobiology of itch. *Nat Rev Neurosci* 7:535–47

Imamachi N, Park GH, Lee H *et al.* (2009) TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. *Proc Natl Acad Sci USA* 106:11330–5

Johanek LM, Meyer RA, Hartke T *et al.* (2007) Psychophysical and physiological evidence for parallel afferent pathways mediating the sensation of itch. *J Neurosci* 27:7490–7

Kempkes C, Buddenkotte J, Cevikbas F *et al.* (2014)Role of PAR-2 in Neuroimmune Communication and Itch. In: *Itch: Mechanisms and Treatment*. Carstens E, Akiyama Teds Boca Raton: (FL)(FL)

Liu Q, Tang Z, Surdenikova L *et al.* (2009) Sensory neuron-specific GPCR Mrgprs are itch receptors mediating chloroquine-induced pruritus. *Cell* 139:1353–65

Moram MM, Fanger C, Chong JA *et al.* (2007) International application published under the patent cooperation treaty (PCT). WO 2007/073505 A2: 1–266

Namer B, Carr R, Johanek LM *et al.* (2008) Separate peripheral pathways for pruritus in man. *J Neurophysiol* 100:2062–9

Oh MH, Oh SY, Lu J *et al.* (2013) TRPA1-dependent pruritus in IL-13-induced chronic atopic dermatitis. *J Immunol* 191:5371–82

Phan NQ, Bernhard JD, Luger TA *et al.* (2010) Antipruritic treatment with systemic mu-opioid receptor antagonists: a review. *J Am Acad Dermatol* 63:680–8

Reddy VB, Iuga AO, Shimada SG *et al.* (2008) Cowhage-evoked itch is mediated by a novel cysteine protease: a ligand of protease-activated receptors. *J Neurosci* 28:4331–5

Reddy VB, Shimada SG, Sikand P *et al.* (2010) Cathepsin S elicits itch and signals via protease-activated receptors. *J Invest Dermatol* 130:1468–70

Rudd JA, Ngan MP, Wai MK (1999) Inhibition of emesis by tachykinin NK1 receptor antagonists in Suncus murinus (house musk shrew). *Eur J Pharmacol* 366:243–52

Russell FA, Zhan S, Dumas A *et al.* (2011) The pronociceptive effect of proteinase-activated receptor-4 stimulation in rat knee joints is dependent on mast cell activation. *Pain* 152:354–60

Shelley WB, Arthur RP (1955) Studies on cowhage (Mucuna pruriens) and its pruritogenic proteinase, mucunain. *AMA Arch Derm* 72:399–406

Steagall RJ, Sipe AL, Williams CA *et al.* (2012) Substance P release in response to cardiac ischemia from rat thoracic spinal dorsal horn is mediated by TRPV1. *Neuroscience* 214:106–19

Steinhoff M, Bienenstock J, Schmelz M *et al.* (2006) Neurophysiological, neuroimmunological, and neuroendocrine basis of pruritus. *J Invest Dermatol* 126:1705–18

Steinhoff M, Neisius U, Ikoma A *et al.* (2003) Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. *J Neurosci* 23: 6176–80

Sun YG, Chen ZF (2007) A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. *Nature* 448:700–3

Sun YG, Zhao ZQ, Meng XL *et al.* (2009) Cellular basis of itch sensation. *Science* 325:1531–4

Tey HL, Yosipovitch G (2011) Targeted treatment of pruritus: a look into the future. *Br J Dermatol* 165:5–17

Tominaga M, Ogawa H, Takamori K (2009) Histological characterization of cutaneous nerve fibers containing gastrin-releasing peptide in NC/Nga mice: an atopic dermatitis model. *J Invest Dermatol* 129:2901–5

Tsujii K, Andoh T, Lee JB *et al.* (2008) Activation of proteinase-activated receptors induces itch-associated response through histamine-dependent and -independent pathways in mice. *J Pharmacol Sci* 108:385–8

Tsujii K, Andoh T, Ui H *et al.* (2009) Involvement of tryptase and proteinase-activated receptor-2 in spontaneous itch-associated response in mice with atopy-like dermatitis. *J Pharmacol Sci* 109:388–95

Vergnolle N (2009) Protease-activated receptors as drug targets in inflammation and pain. *Pharmacol Ther* 123:292–309

Weisshaar E, Apfelbacher C, Jager G *et al.* (2006) Pruritus as a leading symptom: clinical characteristics and quality of life in German and Ugandan patients. *Br J Dermatol* 155:957–64

Willcockson HH, Chen Y, Han JE *et al.* (2010) Effect of genetic deletion of the vanilloid receptor TRPV1 on the expression of Substance P in sensory neurons of mice with adjuvant-induced arthritis. *Neuropeptides* 44: 293–7

Wilson SR, Gerhold KA, Bifolck-Fisher A *et al.* (2011) TRPA1 is required for histamine-independent, Mas-related G protein-coupled receptor-mediated itch. *Nat Neurosci* 14:595–602

Wilson SR, Nelson AM, Batia L *et al.* (2013) The ion channel TRPA1 is required for chronic itch. *J Neurosci* 33:9283–94